

**This Page Is Inserted by IFW Operations  
and is not a part of the Official Record**

## **BEST AVAILABLE IMAGES**

**Defective images within this document are accurate representations of  
the original documents submitted by the applicant.**

**Defects in the images may include (but are not limited to):**

- **BLACK BORDERS**
- **TEXT CUT OFF AT TOP, BOTTOM OR SIDES**
- **FADED TEXT**
- **ILLEGIBLE TEXT**
- **SKEWED/SLANTED IMAGES**
- **COLORED PHOTOS**
- **BLACK OR VERY BLACK AND WHITE DARK PHOTOS**
- **GRAY SCALE DOCUMENTS**

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

UNITED STATES PATENT AND TRADEMARK OFFICE

DEPARTMENT OF COMMERCE

THIS PAGE BLANK (USPTO)



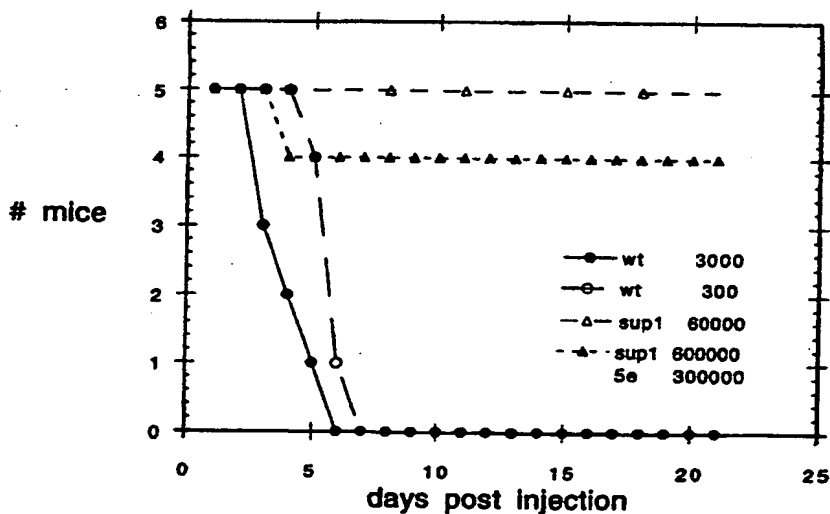
PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>A61K 39/12, 35/76, C12N 7/04, 7/08, C07K 14/03</b>		<b>A1</b>	(11) International Publication Number: <b>WO 97/04804</b>
			(43) International Publication Date: 13 February 1997 (13.02.97)
(21) International Application Number: <b>PCT/US96/12281</b>		(81) Designated States: AL, AM, AU, BB, BG, BR, CA, CN, EE, FI, GE, HU, IS, JP, KG, KP, KR, LK, LR, LT, LU, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TR, TT, UA, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 25 July 1996 (25.07.96)			
(30) Priority Data: 60/001,602 27 July 1995 (27.07.95) US			
(71) Applicant: AMERICAN CYANAMID COMPANY [US/US]; Five Giralda Farms, Madison, NJ 07940 (US).			
(72) Inventors: MOHR, Ian, J.; 2400 Hudson Terrace, Fort Lee, NJ 07024 (US). GLUZMAN, Yakov (deceased).			
(74) Agents: BARNHARD, Elizabeth, M.; American Home Products Corporation, Patent Law Dept. - 2B, One Campus Drive, Parsippany, NJ 07054 (US) et al.			
		<b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	

(54) Title: AVIRULENT HERPETIC VIRUSES USEFUL AS TUMORICIDAL AGENTS AND VACCINES



(57) Abstract

Isolated tumoricidal herpetic viruses, in particular neurotrophic herpes viruses, T-lymphotrophic viruses, and B-lymphotrophic viruses, which are avirulent and capable of selectively replicating in and destroying neoplastic cells, and pharmaceutical compositions, vaccines, and methods of destroying neoplastic cells employing the isolated tumoricidal herpetic viruses are described. A method of isolating tumoricidal herpetic viruses by sequentially passaging attenuated, avirulent herpetic viruses on neoplastic cells which fail to support replication of the herpetic viruses and isolating the viruses which grow on the neoplastic cells is also described. Herpes simplex virus mutants having a genome from which the  $\gamma 34.5$  genes have been deleted and which require at least one additional mutation to produce a non-neurovirulent herpes simplex virus which selectively replicates in and destroys neoplastic cells are also described.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

## **AVIRULENT HERPETIC VIRUSES USEFUL AS TUMORICIDAL AGENTS AND VACCINES**

### **TECHNICAL FIELD**

This invention relates to a method of isolating herpetic viruses, in particular neurotrophic herpes viruses, T-lymphotrophic viruses, and B-lymphotrophic viruses, which are avirulent and capable of replicating in and destroying neoplastic cells, by sequentially passing attenuated, avirulent herpetic viruses on neoplastic cells which fail to support replication of the avirulent herpetic viruses and isolating the viruses which grow on the neoplastic cells. The invention further relates to pharmaceutical compositions, vaccines, and methods of destroying neoplastic cells employing the isolated herpetic viruses.

### **BACKGROUND OF THE INVENTION**

Viruses are obligate intracellular parasites which have evolved highly complex and specialized relationships with their hosts. While some viruses can establish relatively asymptomatic, persistent infections, others are more virulent and infection results in significant morbidity. Because viruses are specialized assemblies which rely on their host cells for replication and dissemination of progeny, the viruses must therefore ensure that the host they have chosen will survive. It has become increasingly clear that many viruses actually encode functions which may foster the survival of the infected cell. The length of survival time will be quite variable, and responsive to external environmental parameters and the fitness of the host. In the case of more virulent viruses, it is only important to ensure host survival long enough to permit the replication and spread of infectious virus. In the absence of dominant viral survival functions, death programs intrinsic to the host cell become activated and the infectious

- 2 -

process is effectively aborted. Development of novel modes of antiviral chemotherapy and the modulation of viral virulence are dependent upon a detailed understanding of this process.

5 The parameters which govern virulence have long been recognized to be of medical importance. The observation by Jenner in the late 1700's that milkmaids exposed to cowpox failed to contract smallpox led him to examine the use of cowpox virus as a vaccine against smallpox. Unbeknownst to him at the time, the key components responsible for the success of this approach were the  
10 high degree of similarity in the overall antigenicity of the two related viruses, and the fact that cowpox was significantly less virulent than its smallpox cousin. The molecular basis for this difference in virulence remains a mystery to this day. Events in more recent history involve the isolation of attenuated viruses which lack the virulence  
15 properties of their wild-type counterparts. These live virus preparations can be used as vaccines, as they present a relatively normal display of viral antigens in the context of mild viral infection. Both of these strategies effectively led to the elimination of smallpox and a great decrease in the incidence of polio.

20 In addition to their use as vaccine strains, there have been reports of tumoricidal effects of viral infections which date back to the turn of the century. These observations suggest that malignant lesions could regress in response to viral infection. The use of viruses as treatments for malignancies, however, must involve the isolation of  
25 strains with selective virulence. To be effective in treatments of malignancies, such isolates should only grow productively and exhibit virulence in neoplastic cells, and should not be capable of propagating a productive infection through surrounding normal, terminally differentiated tissue. As virulence is governed by specific viral genes,  
30 one approach would be to try to genetically alter the virulence of viruses to obtain viruses which selectively destroy neoplastic cells.

- 3 -

Cancer is a proliferative disease whereby neoplastic cells, which have their derivation from a single malignant cell, grow in an unregulated manner and spread throughout the body. In addition to the surgical removal of isolated tumors, current therapies to treat the various forms of cancer revolve around the enhanced sensitivity of malignant cells to a variety of toxic agents. This reflects the fact that the rapidly dividing, undifferentiated neoplastic cells display a heightened sensitivity to the killing potentiated by radiation and chemicals. Normal, terminally differentiated cells are more resistant to the damage or are, perhaps, more proficient at repairing damage to their DNA. Recent advances in the fields of radiotherapy and chemotherapy have involved attempts to improve on the selective targeting of radioligands or cytotoxic reagents to neoplastic cells.

The current modalities of treatment, surgery, radiotherapy, and chemotherapy, have made little impact on the prognosis of some cancers such as gliomas. Gliomas comprise the most common class of human brain tumors. Non-resectable, aggressively growing brain lesions such as glioblastoma, the glioma that occurs with greatest frequency, prove fatal in the vast majority of cases. What is needed to combat gliomas is an avirulent virus which can be delivered to diseased tissue and can selectively replicate in and destroy malignant cells while sparing surrounding normal neural tissue. Such a treatment requires the use of a neurotrophic virus with radically attenuated virulence properties which is still capable of infecting the central nervous system. As Herpes simplex virus-1 (HSV-1) can replicate in the brain, this virus could be useful as a tumoricidal agent.

As an  $\alpha$ -herpesvirus member of the family *Herpesviridae*, HSV-1 infection initiates in the oral cavity. While many primary infections are asymptomatic, limited replication in this peripheral, epithelial tissue ensues, followed by production of a cellular immune

- 4 -

response. Some of the virus produced, however, migrates down axons which innervate this tissue and colonizes the neuronal cell bodies of the trigeminal ganglion. The virus persists here in a latent immunologically cloaked form for the life of the infected individual. Periodically, in response to stress or exposure to ultraviolet light, productive infection ensues in these neurons and mature virions migrate back down axons to the oral epithelia. A productive infection, or reactivation event, is initiated. The neutralization of virus produced in this episode and the fluid which accumulates form a fever blister or cold sore. This mild, benign condition afflicts millions world-wide. However, HSV-1 can also initiate a productive infection of the central nervous system. With a frequency of approximately 1 in 250,000, HSV-1 virions can reach the brain where infection can cause fatal encephalitis.

Neurovirulence of HSV-1 has been shown to be affected by mutations in a number of HSV open reading frames. Martuza in the published European Patent Application No. 0 514 603 describes the use of an HSV-1 mutant, *d/sptk*, as a means to destroy glioblastoma cells. A serious drawback of the *d/sptk* HSV-1 virus mutant is that this HSV-1 virus mutant can still cause fatal encephalitis. See, Markert, J.M., et al., *Neurosurgery*, 32:597-603 (1993); Chambers, R., et al., *Proc. Natl. Acad. Sci. USA*, 92:1411-1415 (1995).

A different type of mutation affecting neurovirulence has been described in Roizman U.S. Patent No. 5,328,688 ("Roizman"), which is incorporated by reference herein in its entirety. Roizman describes HSV-1 mutants having mutations in both copies of the  $\gamma_134.5$  gene to prevent the encoding of an active  $\gamma_134.5$  gene product. Roizman's HSV-1 mutants are greatly attenuated in their neurovirulence properties. However, Roizman's  $\gamma_134.5$  mutant viruses fail to grow in neuronal tumor tissue, making them poor candidates for



- 5 -

a tumor specific agent capable of killing tumor cells. Chambers, R., et al., Proc. Natl. Acad., Sci. USA, 92:1411-1415 (1995). Roizman did find that one  $\gamma_134.5$  mutant virus designated R4009 appeared to be more efficient than his other mutants in destroying neuronal tumor cells. R4009 inserts a stop codon in all three reading frames of the  $\gamma_134.5$  open reading frame. The enhanced destruction of neuronal tumor tissue exhibited by R4009 can thus be due to a low level of  $\gamma_134.5$  expression by "reading through" these stop codons.

Chambers, R., et al., Proc. Natl. Acad., Sci. USA, 92:1411, 1415 (1995). This partial reconstruction of the wild-type phenotype illustrates the potential dangers of utilizing viruses which contain point mutations as therapeutic agents. Viruses which contain point mutations that impair their growth may eventually revert to wild-type and cause encephalitis.

In PCT Publication No. WO 96/00007, Martuza combined a ribonucleotide reductase mutation with Roizman's  $\gamma_134.5$  deletion mutant virus designated R3616. Essentially, Martuza's approach does not differ from the studies described by Chambers, R., et. al., Proc. Natl. Acad. Sci. USA, 92: 1411-1415 (1995). Like Roizman's  $\gamma_134.5$  mutant virus described above, this double mutant virus would not be effective as an antineoplastic agent because this double mutant virus grows poorly in tumor tissue. Indeed, the ribonucleotide reductase mutation combined with the  $\gamma_134.5$  mutation does not repair the profound growth defect of  $\gamma_134.5$  mutant viruses on tumor tissue. To the contrary, this additional mutation serves to further cripple the virus, as evidenced by the enhanced sensitivity of this double mutant virus to gancyclovir. Another serious disadvantage of this double mutant virus is that the ribonucleotide reductase mutation is an insertion which could easily revert to yield a virus which contains only a  $\gamma_134.5$  mutation. While a body of evidence exists supporting the notion that  $\gamma_134.5$  mutant viruses are sufficiently attenuated to

- 6 -

warrant safe intracranial administration, they are limited by the inability to grow efficiently and destroy neoplastic cells. It would thus be highly desirable to create a virus which retained the attenuation properties of the  $\gamma$ 34.5 mutant virus, but which would display selective, robust growth on malignant cells.

Accordingly, an object of the present invention is to provide viruses that are avirulent and can selectively replicate in tumor tissue, thereby having the capabilities of destroying a localized mass of tumor cells and sparing the surrounding normal tissue. A further object of the present invention is to provide a method of treatment of tumors employing these viruses. Another object of the present invention is to provide a vaccine protecting against herpes infections. These and other objects and features of the invention will be apparent from the following description.

### **SUMMARY OF THE INVENTION**

The present invention provides a herpetic virus selected from the group consisting of neurotrophic herpes viruses, T-lymphotrophic viruses, and B-lymphotrophic viruses having a genome from which the gene or genes controlling virulence have been deleted and which requires at least one additional mutation to the genome, wherein the mutated herpetic virus is avirulent and selectively replicates in and destroys neoplastic cells. When the herpetic virus is a herpes simplex virus, the genes controlling virulence that are deleted are both copies of the  $\gamma$ 34.5 gene. Surprisingly, these herpetic viruses are both avirulent and able to selectively replicate in neoplastic cells. A pharmaceutical composition containing the herpetic virus mutant of the present invention is also provided.

An advantage of the herpetic virus mutant is that the virus destroys neoplastic cells without productively infecting surrounding normal, differentiated cells and generating a fatal infection

- 7 -

of the central nervous system.

The present invention also provides a method for selectively destroying neoplastic cells in a mammal by administering to the mammal an effective amount of the herpetic virus of the present invention.

An advantage of this method is that an effective amount of the herpetic virus may be administered to a mammal to destroy neoplastic cells in the mammal without the danger of generating a fatal infection of the central nervous system.

The present invention further provides a vaccine for immunizing a mammal against herpetic viruses comprising the herpetic virus of the present invention and a pharmaceutically acceptable diluent, adjuvant or carrier, as well as a method of immunizing a mammal employing this vaccine.

An advantage of this vaccine is that the herpetic virus cannot cause a productive infection of the central nervous system of the recipient.

The present invention still further provides a method for isolating tumoricidal herpetic viruses by attenuating a herpetic virus to render it avirulent; identifying neoplastic cells which fail to support replication of the avirulent virus; sequentially passaging the attenuated virus on the neoplastic cells to produce isolates of the attenuated virus which can replicate in the neoplastic cells; and isolating the resulting viruses.

An advantage of this method is that the separation of the genetic neurovirulence determinants from the functions which enable the virus to replicate in neoplastic cells enables the selection of viruses which are avirulent and able to replicate in tumor cells, but not in normal, terminally differentiated cells.

Upon further study of the specification and appended claims, further objects and advantages of this invention will become

- 8 -

apparent to those skilled in the art.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

FIG. 1 is a schematic of the replacement of 34.5 coding sequences of HSV-1 with the  $\beta$ -glucuronidase gene. Letter abbreviations for restriction endonuclease cleavage sites are B = *Bam*HI, S = *Sac*I, and N = *Nco*I. U<sub>L</sub> = Unique Long region of HSV-1. U<sub>S</sub> = Unique Short region of HSV-1. The shaded region in the targeting construct at the 3' end of the  $\beta$ -glucuronidase gene represents the human cytomegalovirus Us10 polyadenylation site.

FIG. 2 is a schematic of the isolation of second-site suppressor mutants from parental SPBg5e 34.5 deletion ( $\Delta$ 34.5) stocks.

FIG. 3 is a drawing presenting results of a growth analysis of a selected suppressor isolate. pfu = plaque forming units.  $\Delta$ 34.5 = SPBg5e virus. wt = wild-type HSV-1 Patton strain. REV 4 = a suppressor isolate.

FIG. 4 is an autoradiograph of a Southern blot detailing the genetic structure of the *Bam*HI Z fragment of suppressor isolates 1 to 8 and 10 and REV4; 34.5 deletion mutant SPBg53e; and wild-type (W.T.) HSV-1 (Patton strain).

FIG. 5 is a drawing highlighting the junction between the Unique Short region and the Short Terminal Repeats of the *Bam*HI Z fragment in wild-type HSV-1.

FIGS. 6A-D are drawings which identify the nucleotide sequences in the *Bam*HI Z fragment where the nucleotide sequences of the suppressor isolates deviate from the nucleotide sequences of wild-type HSV-1 (Patton strain). All the nucleotide numbers in FIGS. 6A-D correspond to the nucleotide numbers of the published sequence of strain 17, GENBANK accession number X14112D00317. FIG. 6A shows that for suppressor isolates SUP1 and SUP10, a deletion of

- 9 -

583 base pairs occurs in the *Bam*HI Z fragment between nucleotide 145415 and nucleotide 145999. The base pairs flanking the deletion on the left are hereinafter designated SEQ ID NO: 1, and the base pairs flanking the deletion on the right are hereinafter designated SEQ ID NO: 2. FIG. 6B shows that for suppressor isolates SUP5 and SUP8, a deletion of 497 base pairs occurs in the *Bam*HI Z fragment between nucleotide 145421 and nucleotide 145919. The base pairs flanking the deletion on the left are hereinafter designated SEQ ID NO: 3, and the base pairs flanking the deletion on the right are hereinafter designated SEQ ID NO: 4. FIG. 6C shows for suppressor isolates SUP2 and SUP6 the occurrence of insertions of repetitive DNA elements along with deletions of genetic material. The base pairs on the left of checkboard rectangles are hereinafter designated SEQ ID NO: 5. There are two SUP6 in FIG. 6 because two SUP6 clones were obtained from one suppressor isolate. The checkerboard rectangles represent an iteration of HSV-1 sequences normally present from nucleotide numbers 145462 to 145477, hereinafter designated SEQ ID NO: 6. The small shaded oval represents HSV-1 sequences having nucleotide numbers 145481-145491, hereinafter designated SEQ ID NO: 7. The elongated shaded oval represents sequences which share homology to HSV-1 sequences contained in the repetitive portion of the viral genome. FIG. 6D shows for suppressor isolate SUP3 the occurrence of insertions of repetitive DNA elements along with deletions of genetic material. The elongated striped oval represents sequences which share homology to HSV-1 sequences contained in the repetitive portion of the viral genome. The base pairs to the left of the elongated striped oval are hereinafter designated SEQ ID NO: 8.

FIG. 7 is an autoradiograph of an SDS polyacrylamide gel which displays proteins synthesized at late times post-infection by selected suppressor isolates on U373 human glioblastoma cells. Lane 1 is uninfected U373 human glioblastoma cells (U373). Lane 2 is

- 10 -

SPBg5e virus (MUT). Lane 3 is suppressor isolate SUP10. Lane 4 is suppressor isolate SUP4. Lane 5 is suppressor isolate REV4. Lane 6 is wild-type HSV-1 Patton strain (W.T.). Lane 7 is molecular weight markers (M).

5                   FIG. 8 is a graph displaying survival results of mice injected intracranially with different amounts of suppressor isolate SUP1, SPBg5e  $\Delta$ 34.5 mutant (5e), or wild-type HSV-1 Patton strain (wt). • = 3,000 pfu wild-type HSV-1 Patton strain. ○ = 300 pfu wild-type HSV-1 Patton strain.  $\Delta$  = 60,000 pfu suppressor isolate SUP1. 10                   ▲ = 300,000 pfu SPBg5e  $\Delta$ 34.5 mutant virus and ◆ = 600,000 pfu suppressor isolate SUP1, as the data was identical for each.

FIG. 9 is a schematic of the experimental technique used to rescue the suppressor phenotype of the second-site suppressor mutants.

15                   FIG. 10 is an autoradiograph of a Southern blot detailing the genetic structure of the *Bam*HI Z fragment of the rescued suppressor isolates 1, 3, 5, and 6 (SUP1, SUP3, SUP5, and SUP6) and of the 34.5 deletion mutant SBg53e ( $\Delta$ 34.5). The lanes labelled with a "C" contained cloned plasmid DNA used in the marker rescue 20                   transfection which was subsequently digested with *Bam*HI. The lanes labelled with a "V" contain *Bam*HI digested viral DNA recovered after passage of the transfection lysate on U373 cells.

### DETAILED DESCRIPTION OF THE INVENTION

25                   It is to be understood that while the present invention is primarily contemplated for humans, it is also contemplated for use in veterinary medicine.

30                   The present invention provides a novel method for isolating herpetic viruses that are both avirulent and can replicate in neoplastic cells, but not in normal, differentiated cells, thereby having the capability to destroy a localized mass of neoplastic or tumor cells.

- 11 -

Prior to the present invention, viruses have been rendered avirulent by different techniques. However, either the resulting avirulent viruses could not grow in tumor cells and therefore could not be used to destroy the tumor cells, or they were not attenuated sufficiently and were still capable of generating a productive infection in the central nervous system. For the first time, the functions of virulence and the ability to grow on tumor cells in a herpetic virus have been genetically separated and an avirulent herpetic virus has been produced which can grow in tumor cells.

In the method for isolating tumoricidal herpetic viruses, the herpetic virus is attenuated to render it avirulent. The herpetic virus is preferably a neurotrophic virus, a B-lymphotrophic virus, or a T-lymphotrophic virus. The neurotrophic virus is preferably a herpes simplex virus and is more preferably HSV-1, HSV-2, or Varicella-zoster virus (VZV). The T-lymphotrophic virus is preferably human herpes virus-6 (HHV-6). The B-lymphotrophic virus is preferably Epstein-Barr virus. The herpetic virus is rendered avirulent by altering the gene or genes controlling virulence using techniques known in the art which include point mutations (substitutions), insertions or deletions to the gene or genes. It is preferred to render the herpetic virus avirulent by completely deleting the gene or genes controlling virulence to minimize the possibility of reversion to a virulent form of the wild-type virus. When the herpetic virus is a herpes simplex virus, both copies of the  $\gamma$ 34.5 gene are completely deleted.

The next step in the method is to identify a neoplastic cell that will not support replication or will support, at most, limited replication of the avirulent herpetic virus as compared to replication of the wild-type herpetic virus in the neoplastic cell. Such neoplastic cells include cells of tumors, carcinomas, sarcomas, leukemia, lymphomas, and the like. Nervous system tumors include astrocytomas, oligo-dendrogliomas, meningiomas, neurofibromas,

- 12 -

ependymomas, Schwannomas, neurofibrosarcomas, and gliomas such as glioblastomas.

Once the neoplastic cell is identified, the avirulent virus is sequentially passaged on the identified neoplastic cell until viral isolates are produced which display efficient, productive growth compared to the starting avirulent parent virus (which does *not* grow in neoplastic cells). The sequential passaging is carried out by infecting neoplastic cells either just confluent or approaching confluence with avirulent virus at multiplicities of infection of  $10^{-1}$  to  $10^{-4}$ . Cultures are examined each day for signs of cytopathic effect. When the cultures displayed either complete cytopathic effect or the neoplastic cells have completely exhausted the media, a lysate is prepared by freeze-thawing and sonicating for approximately one minute in a water bath. A second culture of neoplastic cells is infected with 0.1 ml of the lysate and the entire passaging process repeated four times in succession. Those cultures which are capable of generating substantial cytopathic effect on the neoplastic cells prior to the cells exhausting the media are isolated and subjected to two rounds of plaque purification. Large scale stocks are then prepared on the neoplastic cells.

Only those avirulent viruses that have regained the ability to grow on the identified neoplastic cell will replicate in those neoplastic cells and form isolates from which these viruses can be obtained. To regain the ability to grow on the identified neoplastic cell, at least one additional mutation must occur to the genome of the herpetic virus. When the avirulent virus is a herpes simplex virus, at least one of the additional mutations to the gene may affect the nucleotide sequence between *Bst* EI (corresponding to nucleotide number 145316 of the published sequence of strain 17, GENBANK accession number X14112D00317) and *Nco* I (corresponding to nucleotide number 146592 of the published sequence of strain 17,



- 13 -

GENBANK accession number X14112D00317) of the *Bam*HI Z fragment of HSV-1. As this isolate has sustained a mutation at a site distinct from the  $\gamma$ 34.5 genes, it is a second-site suppressor mutant, also referred to as a suppressor isolate, which rescues the growth defect of  $\gamma$ 34.5 deletion viruses on neoplastic neuronal cells.

A sample of a HSV-1 suppressor isolate in accordance with the present invention, designated SUP1, has been deposited under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC) at 12301 Parklawn Drive, Rockville, MD 20852 USA under Accession Number ATCC VR-2510.

The isolated avirulent herpetic virus is used to destroy neoplastic cells in a mammal because of its ability to infect and replicate in such neoplastic cells. Administration of the herpetic viral isolate to a mammal in an effective amount provides circulating virus which enters a mass of malignant tumor cells and initiates and propagates an infection in the malignant cells which results in their destruction. Alternatively, the herpetic viral isolate is injected into the mammal at or near the site of neoplastic growth. The amount of virus to be administered ranges in concentration from about  $10^1$  to about  $10^{10}$  plaque forming units (pfu), preferably from about  $5 \times 10^4$  pfu to  $1 \times 10^6$  pfu, and more preferably from about  $1 \times 10^5$  to about  $4 \times 10^5$  pfu, although the most effective ranges may vary from host to host. When the neoplastic cells to be destroyed are glioma cells, the preferred isolated herpetic virus is a herpes simplex virus such as HSV-1 or HSV-2 having both copies of the  $\gamma$ 34.5 gene deleted and an additional mutation enabling the herpes simplex virus to selectively replicate in the glioma cells. The HSV-1 suppressor isolate (SUP1) deposited under ATCC Accession Number VR-2510 is used in this method of destroying neoplastic cells.

A pharmaceutical composition containing the isolated herpetic virus is used for treating tumors in a mammal. The

- 14 -

pharmaceutical composition comprises the isolated herpetic virus of the present invention and a pharmaceutically acceptable carrier, adjuvant or diluent. The pharmaceutical composition may be in injectable form.

5                   The isolated herpetic viruses of the present invention are also employed as a vaccine against herpetic viruses when combined with a pharmaceutically acceptable diluent, adjuvant, or carrier. A mammalian host, preferably human, is inoculated with a vaccine comprising an immunity-inducing dose of one or more of the live  
10                   vaccinal herpetic viral strains of the invention by the parenteral route, preferably by intramuscular or subcutaneous injection. Inoculation may also be effected by surface scarification, or by inoculation of a body cavity. Typically, one or several inoculations of between about 10 and 1,000,000 pfu each, as measured in susceptible human or  
15                   nonhuman primate cell lines, are sufficient to effect immunization of a human host.

                  The vaccine may be conveniently utilized in liquid form or in freeze-dried form, in the latter case in combination with one or more suitable preservative and protective agents to protect the vaccinal  
20                   strains during the freeze-drying process.

                  In a preferred embodiment, a recombinant HSV-1 virus (strain Patton) was created where both copies of the  $\gamma$ 34.5 gene were replaced by DNA sequences encoding  $\beta$ -glucuronidase, as illustrated by FIG. 1. Briefly, FIG. 1 shows a schematic drawing of the HSV-1  
25                   genome highlighting the locations of both copies of the  $\gamma$ 34.5 gene in a repetitive region of the genome. The targeting construct shown in FIG. 1 is a plasmid which targets the  $\beta$ -glucuronidase gene to the 34.5 genetic locus. The plasmid was created by cloning the HSV-1 sequences which normally bracket the 34.5 gene into the  
30                   corresponding positions so as to surround the  $\beta$ -glucuronidase gene. After cotransfecting the targeting construct along with wild-type HSV-

- 15 -

1 DNA onto Vero cells by the calcium-phosphate precipitate (Ca-phosphate ppt.) technique, recombinant virus, designated the parental SPBg5e virus, was isolated by plaque purification in the presence of indicator dye or colorimetric substrate to facilitate the identification of recombinant plaques on Vero cells.

SKNSH neuroblastoma cells, obtained from the ATCC, are non-permissive for the growth of 34.5 HSV-1 mutants. Presumably, this reduction in viral growth reflects the fact that all protein synthesis ceases with the onset of viral DNA replication. See, Chou, J. and Roizman, B., Proc. Natl. Acad. Sci. USA, 89:3266-3270 (1992). The recombinant HSV-1 mutant designated SPBg5e also failed to synthesize wild-type levels of late proteins on SKNSH cells. The premature termination of protein synthesis in neuroblastoma cells infected with this 34.5 deletion mutant causes a dramatic reduction in viral yield. FIG. 3 shows the difference in yield between the 34.5 deletion mutant ( $\Delta$ 34.5) and the wild-type (wt) virus. For example, at 100 pfu, the difference in yield is 56,000 fold. This growth defect was employed as and found to be a powerful means for biological selection to isolate second-site suppressor mutants.

The parental SPBg5e virus, which completely lacked all coding sequences for the 34.5 gene, was passaged multiple times in succession on SKNSH neuroblastoma cells, as illustrated in FIG. 2. Multiple plaque-purified independent isolates were capable of sustained protein synthesis and growth on cells of neuronal origin, notably SKNSH neuroblastoma and U373 human glioblastoma (see FIGS. 3 and 7). Figure 3 is a schematic outlining the procedure to analyze the growth of selected suppressor isolates on SKNSH cells at different multiplicities of infection. Figure 3 shows as an example, either 10, 100, or 1,000 pfu of wild-type HSV-1,  $\Delta$ 34.5 mutant (SPBg5e), or REV4 (a suppressor isolate in accordance with the present invention) were used to infect SKNSH cells. After incubating

- 16 -

48 hours at 37°C, a lysate was obtained by freeze thawing and the viral titer was determined on Vero cells. At all multiplicities of infection examined, the suppressor isolate REV4 grew 50 to 100 times better than the parental  $\Delta$ 34.5 deletion mutant on SKNSH cells. Analysis of viral DNA revealed that all of the suppressors rearranged a region of the *Bam*HI Z fragment of HSV-1 (Patton strain) where the Unique Short component joins the Short Terminal Repeat. These mutations affect the cis-acting sequences which direct transcription of the Us11 open reading frame and disrupt the coding region of the Us12 open reading frame (see FIG. 5). Analysis of viral proteins produced late in infection confirmed that the Us11 protein is either not produced or is produced at enormously reduced levels (see FIG. 7).

The genetic rearrangements most likely affect one or more components of a signaling pathway which involves the protein encoded by the  $\gamma$ 34.5 gene. In the absence of the 34.5 gene product, the virus presumably generates a signal which leads to the cessation of protein synthesis. This inhibition of translation leads to the death of the infected cell prior to the assembly of optimal amounts of infectious progeny. The  $\gamma$ 34.5 gene encodes a factor which fosters the survival of the infected cell and the assembly and dissemination of infectious virus. The combined absence of the signal which potentiates the shutdown of translation and the deletion of the 34.5 neuronal survival function creates a novel virus which can efficiently replicate in malignant glioblastoma tissue, but does not cause fatal encephalitis. As all 34.5 coding sequences are deleted in the viruses of this embodiment of the invention, it is not possible to reconstruct the wild-type phenotype by producing small amounts of the 34.5 gene product, thereby avoiding infection of the central nervous system. Novel genetic changes have occurred in these isolates which restore the ability of the virus to grow productively on neoplastic cells. The method of the present invention results in the separation of th

- 17 -

genetic neurovirulence determinants from the functions which enable the virus to replicate in neoplastic cells.

5 The present invention demonstrates for the first time that it is possible for a non-neurovirulent herpetic virus unable to productively grow on neoplastic cells to reacquire the ability to productively grow on neoplastic cells without reacquiring a neurovirulent phenotype. When herpes simplex virus has a single genetic element, the  $\gamma 34.5$  gene, deleted, the herpes simplex virus loses its neurovirulence, but also loses the ability to replicate in  
10 neoplastic neuronal cells SKNSH and U373. The present invention has solved this problem by providing a novel herpes simplex virus from which the  $\gamma 34.5$  gene has been deleted and which contains a mutation in a different genetic element that restores the virus' ability to replicate in neoplastic neuronal cells, including SKNSH and U373  
15 cells, but retains the non-neurovirulent phenotype.

Without being bound by theory, it is believed that inoculation of these 34.5 suppressor isolates intracranially into brains afflicted with glioblastoma results in the selective destruction of malignant tissue. When the virus destroys a mass of tumor cells and  
20 encounters normal brain tissue, a self-limiting infection is initiated at this peripheral zone which curtails the spread of infectious virus. As these isolates can replicate in glioblastoma cells, a limiting feature of the 34.5 deletion mutants described by Roizman, which do not replicate in glioblastoma cells, has been overcome. Since the  
25 suppressor isolates replicate efficiently in glioblastoma cells, small amounts of circulating virus which enter a mass of malignant cells are able to initiate and propagate an infection solely in the malignant cells. One possible explanation for the suppressor isolates' ability to replicate in glioblastoma cells is that the suppressor mutants are now  
30 dependent on the proliferative capacity of their host cell, and have lost the ability to grow efficiently on cells which are terminally

- 18 -

differentiated. Since malignant cells have shed their terminally differentiated phenotype, they are now suitable hosts for the growth of the suppressor mutants.

5 In the event that viral titers in tumor masses reach potentially dangerous levels, the infection can be controlled by administering an anti-viral compound such as acyclovir, as all these suppressor viruses have a wild-type thymidine kinase (tk) gene. Their replication activity can be further curtailed by combining mutations in other non-essential replicative functions, for example, ribonucleotide  
10 reductase, into the suppressor genome. This imposes a limit on the titer of replicating virus in the vicinity of the tumor mass and provides additional host specificity for actively dividing cells, as the complementing cellular enzyme is under stringent cell-cycle control.

15 The additional mutation to the suppressor isolates also disrupt the Us12 open reading frame. The protein product encoded by Us12 is the  $\alpha 47$  immediate early polypeptide. As this protein product accumulates at early times post-infection, it would need to have a previously undescribed late function for it to be involved in the premature termination of late protein synthesis. The  $\alpha 47$  polypeptide  
20 has been shown to down regulate expression of cellular class I MHC molecules in infected cells, and as such is an important modulator of the immune system. The fact that cells infected with suppressor isolates display functional MHC class I molecules on their surface implies that viral antigen presentation may be up-regulated in these  
25 cells (relative to those infected with a virus which carries a wild-type allele of  $\alpha 47$ ). The presence of viral antigens on the surface of tumor cells may further facilitate tumor destruction by components of the host immune system.

30 Additionally, these suppressor isolates are useful as potential vaccine strains. Like the parental 34.5 deletion mutants, the suppressor isolates do not infect the central nervous system and

- 19 -

cause encephalitis. Since the suppressor isolates of the present invention replicate better than the parental 34.5 deletion mutants in some cultured cells, it is expected that the suppressor isolates would also replicate better than parental 34.5 deletion mutants in peripheral tissue and thus elicit the requisite immune response capable of inducing productive immunity.

In order that this invention may be better understood, the following examples are set forth. The examples are for the purposes of illustration only and are not to be construed as limiting the scope of the invention.

## **EXAMPLES**

### **EXAMPLE 1**

#### **Replacement of HSV-1 34.5 Coding Sequences With $\beta$ -glucuronidase Gene**

The plasmid pBgdUs10, described in Jones, T., et al., J. Virol., 65:5860-5872 (1991), was digested with *Apal*, end filled with Klenow polymerase, and subsequently digested with *XbaI*. The 2.1 kb fragment which contains the  $\beta$ -glucuronidase gene (available from Clontech, San Diego, California as pRAJ275b) fused to the human cytomegalovirus Us10 polyadenylation site (an *Apal-SmaI* fragment corresponding to human cytomegalovirus strain AD169 nucleotide numbers 13527-13782; GENBANK accession number X04650) at its 3' border, was isolated and cloned into pT7-1 (U.S. Biochemical, Cleveland, Ohio) which had been digested with *XbaI* and *SmaI* to create pBg10pA. The HSV-1 (Patton strain) *BamHI* SP fragment (corresponding to nucleotide numbers 123459-129403 in the published sequence of strain 17, GENBANK accession number X14112D00317), described in Jones, T.R. and Hyman, R.W., Virology, 131:555-560 (1983), was cloned into the *BamHI* site of pBR322 and digested with *NcoI*. The 1.8 kb *NcoI* fragment

- 20 -

(corresponding to nucleotide numbers 127666-125855 in the published sequence of strain 17, GENBANK accession number X14112D00317) which contains the promoter and the first ATG codon of the 34.5 gene was isolated and cloned into *Nco*I digested pBg10pA. This plasmid (p5'γ34.5Bg10pA) was digested with *Eco*RI, end-filled with Klenow polymerase, and then digested with *Sac*I. The HSV-1 *Bam*HI SP clone was digested with *Bam*HI, end filled with Klenow polymerase, and digested with *Sac*I. The 1.6 kb terminal *Sac*I - *Bam*HI (Klenow filled) fragment (corresponding to nucleotide numbers 123459-125066 in the published strain 17 sequence, GENBANK accession number X14112D00317) was isolated and ligated into *Eco*RI (Klenow filled)/ *Sac*I digested p5'γ34.5Bg10pA to create p5'γ34.53'γ34.5, which has been abbreviated as pSPBg. This plasmid places the β-glucuronidase gene under the control of the endogenous 34.5 promoter and surrounds the β-glucuronidase gene with HSV-1 sequences which normally flank the 34.5 gene. Five μg of purified plasmid (linearized with *Hind*III), 2 μg of purified HSV-1 (Patton strain) DNA and sonicated salmon sperm DNA carrier were assembled and introduced into Vero cells by the calcium-phosphate technique. Once the cytopathic effect had progressed throughout the monolayer, a lysate was obtained by freeze-thawing, followed by a one minute burst in a water bath sonicator. Dilutions were prepared and isolates were subjected to two rounds of plaque purification on Vero cells in the presence of the indicator dye X-gluc. The isolate used in subsequent studies was designated SPBg5e. Restriction digestion and Southern analysis demonstrated that the gene for β-glucuronidase had replaced both copies of the 34.5 gene.

## EXAMPLE 2

### Selection Procedure to Obtain Suppressor Mutants

Sixty mm dishes of SKNSH neuroblastoma cells which



- 21 -

5 were either just confluent, or approaching confluence, were placed in DMEM plus 2 % fetal bovine serum (FBS) and infected with SPBg5e at multiplicities of infection of  $10^{-1}$  to  $10^{-4}$ . Cultures were examined each day for signs of cytopathic effect. Generally, the cultures displayed either complete cytopathic effect (at higher multiplicities of infection) or the neuroblastoma cells had completely exhausted the media by five to seven days post infection. At this point, a lysate was prepared by freeze-thawing, sonicated for one minute in a water bath, and 0.1 ml of this lysate was used to infect a second 60 mm dish. This process was repeated four times in succession. At this time, some of the cultures were capable of generating substantial cytopathic effect on SKNSH prior to the cells exhausting the media. Isolates were then subjected to two rounds of plaque purification on Vero cells, and large scale stocks were then prepared on SKNSH cells. Staining of the plaques with X-gluc revealed that the isolated plaques retained the  $\beta$ -glucuronidase gene of the parental virus and were capable of growth on both Vero and SKNSH cells.

### EXAMPLE 3

#### Analysis of Viral DNA From Suppressor Isolates

20 Stocks of suppressor isolates prepared on SKNSH cells prepared by the procedures of Example 2 were used to infect Vero cells at a multiplicity of infection of one. Infected cell cultures were maintained in Media 199 (m199)(Gibco BRL, Bethesda, MD) plus 1% calf serum and incubated at 34°C until maximum cytopathic effect was observed. Cells were harvested, suspended in 10 mM Tris, pH 8.4, 140 mM NaCl, 10 mM  $MgCl_2$ , and lysed by addition of the same buffer containing 1% Triton X-100™. After sitting five minutes on ice, the extracts were centrifuged at 14,000 x g for two minutes. The supernatant (removed with a wide bore tip) was adjusted to a final concentration of 0.4% SDS, 10mM EDTA, 25  $\mu$ g/ml RNase A, 1

- 22 -

mg/ml Pronase and incubated for at least one hour at 37°C. DNA was purified by two extractions with phenol:chloroform, one extraction with chloroform, and then precipitated with ethanol. Following a wash with 70% ETOH, the pellet was allowed to dry at room temperature, and resuspended in 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA. These minipreparations of viral DNA yielded adequate amounts for molecular cloning.

At first, viral DNA was cloned as a series of *EcoRI* fragments into *EcoRI* digested pBR322. Restriction digests and Southern analysis established that the breakpoints appeared to be confined in the *BamHI* Z fragment (corresponding to nucleotide numbers 144875-146828 in the published strain 17 sequence, GENBANK accession number X14112D00317). To subsequently isolate *BamHI* Z fragments, viral DNA was digested with *BamHI* and fragments which migrated between 1 and 3 kb were size selected by agarose gel electrophoresis, purified, and cloned into *BamHI*-digested pBR322. Clones containing *BamHI* Z inserts were identified by colony hybridization, and confirmed by Southern blotting. *BamHI* Z fragments from the suppressor isolates were digested with a variety of single cut enzymes to map the extent of the deletion. As all of the isolates retained the *BstEII* site contained within the unique portion of *BamHI* Z, a sequencing primer was designed to read from this point towards the repetitive region. The sequencing primer had the following sequence:

5' CCCTCCGCCCAGAGACTCG 3'

The sequence of the sequencing primer corresponds to nucleotide numbers 145270-145288 in the published sequence of strain 17 (GENBANK accession number X14112D00317) and is hereinafter designated SEQ ID NO: 9. DNA sequencing was performed using a USB Sequenase™ kit (U.S. Biochemical, Cleveland, Ohio) according to the manufacturer's instructions.

- 23 -

Figure 4 is an autoradiograph of a Southern blot where DNA from suppressor isolates SUP1 to 8, SUP10, and REV4; 34.5 deletion mutant SPBg5e; and wild-type HSV-1 (Patton strain) were prepared as described above in Example 3 and digested with *Bam*HI. After separating the fragments by agarose gel electrophoresis and blotting, the membrane was probed with a <sup>32</sup>P-labeled *Bam*HI-*Bst*EII fragment corresponding to the Unique portion of the *Bam*HI Z fragment (corresponding to nucleotide numbers 144875-145316 in the published sequence of strain 17, GENBANK accession number X14112D00317). Restriction digestion and Southern analysis of each isolate demonstrated that all of the suppressors had a DNA rearrangement. In one isolate (REV4), a complex inversion occurred, while all of the others involved deletions or insertions (see FIG. 4).

The *Bam*HI Z fragment from each of several suppressor isolates, including those identified in Figure 4, was isolated by molecular cloning techniques as described above in Example 3. Although the 440 bp *Bam*HI-*Bst*EII Unique Short piece was wild-type by restriction analysis, it was evident that a rearrangement had occurred in all of the isolates between the *Bst*EII site and the *Eco*RI site. Figure 5 shows a physical map of a segment of the wild-type *Bam*HI Z fragment which begins at the *Bam*HI site in the Unique Short region and extends through the *Nru* site in the Short Terminal Repeats (corresponding to nucleotide numbers 144875-146008 of the published sequence of strain 17, GENBANK accession number X14112D00317). The sequencing primer having the sequence 5' CCCTCCGCCAGAGACTCG 3' (SEQ ID NO: 9) was designed to read from the *Bst*EII site towards the end of the Unique Short sequences. The sequencing was performed as described above. The points at which the sequences of the suppressor isolates deviated in the *Bam*HI Z fragment from wild-type HSV-1 were identified and are displayed in FIG. 6.

- 24 -

Figures 6A-D identify the nucleotide sequences in the *Bam*HI Z fragment where the nucleotide sequences of the suppressor isolates deviate from the nucleotide sequences of wild-type HSV-1 (Patton strain). All the nucleotide numbers in FIGS. 6A-D correspond to the nucleotide numbers of the published sequence of strain 17, GENBANK accession number X14112D00317. As shown in FIG. 6A, a deletion of 583 base pairs occurs in the *Bam*HI Z fragment between nucleotide 145415 and nucleotide 145999 of suppressor isolates SUP1 and SUP10. As shown in FIG. 6B, a deletion of 497 base pairs occurs in the *Bam*HI Z fragment between nucleotide 145421 and nucleotide 145919 of suppressor isolates SUP5 and SUP8. As shown in FIG. 6C, insertions of repetitive DNA elements along with deletions of genetic material occurs in suppressor isolates SUP2 and SUP6. The point at which the nucleotide sequence of the suppressor isolate deviates from the nucleotide sequence of wild-type HSV-1 (Patton strain) in the *Bam*HI Z fragment is nucleotide 145477. There are iterations of HSV-1 sequences normally present from nucleotide numbers 145462 to 145477 (SEQ ID NO: 6); HSV-1 sequences having nucleotide numbers 145481-145491 (SEQ ID NO: 7); and sequences which share homology to HSV-1 sequences contained in the repetitive portion of the viral genome. The nucleotide sequence of the more distal junction is not yet known. As shown in FIG. 6D, insertions of repetitive DNA elements along with deletions of genetic material occur in suppressor isolate SUP3. The point at which the nucleotide sequence of the suppressor isolate deviates from the nucleotide sequence of wild-type HSV-1 (Patton strain) in the *Bam*HI Z fragment is nucleotide 145473. There are sequences which share homology to HSV-1 sequences contained in the repetitive portion of the viral genome. The nucleotide sequence of the more distal junction is not yet known.

Analysis of this sequencing data revealed that all of the

- 25 -

suppressors shared the common characteristic of a rearrangement in a region of the *Bam*HI Z fragment of HSV-1 (Patton strain) where the Unique Short component joins the Short Terminal Repeat. These mutations affect the cis-acting sequences which direct transcription of the Us11 open reading frame and disrupt the coding region of the Us12 open reading frame (see FIG. 5). Analysis of viral proteins produced late in infection confirmed that the Us11 protein is either not produced or is produced at enormously reduced levels (see FIG. 7). The genetic rearrangements most likely affect one or more components of a signaling pathway which involves the protein encoded by the  $\gamma$ 34.5 gene.

#### EXAMPLE 4

##### Analysis of Total Viral Protein Synthesis

U373 human glioblastoma cells, obtained from the ATCC, were infected with either SPBg5e (34.5 deletion mutant), wild-type HSV-1 (Patton strain), or suppressor isolates SUP10 and SUP4 at a multiplicity of infection of approximately ten, or infected with suppressor isolate REV4 at a multiplicity of infection of one for approximately one hour at 37°C. The cells were then refed with DMEM plus 2% FBS and allowed to incubate overnight. At any point after 12 hours post-infection (usually 15.5 hours), the infected cells were overlaid with one ml of DMEM containing 50-70  $\mu$ Ci/ml  $^{35}$ S Express™ (a commercial mixture of methionine and cysteine from DuPont New England Nuclear, Boston, Massachusetts) and the incubation continued for one hour. Total cellular protein was solubilized in 1X Laemli buffer, boiled for three minutes, and a portion was fractionated on 12.5% SDS-polyacrylamide gels. There were also separate lanes in the gel for uninfected U373 cells and for a  $C^{14}$ -labeled high molecular mixture weight (MW) from Amersham as molecular weight markers for comparative purposes. Gels were fixed

- 26 -

in 25% MeOH, 10% acetic acid, dried, and exposed to Kodak XAR film.

5 The results are displayed in Figure 7. Figure 7 is an autoradiograph of an SDS polyacrylamide gel which displays viral proteins produced at late times post infection in U373 cells infected with either the  $\Delta$ 34.5 mutant (MUT), the suppressor isolates SUP10, SUP4, or REV4, or wild-type HSV-1 (W.T.). While infection of U373 cells with the 34.5 deletion mutant results in the cessation of protein synthesis, the suppressor isolates are capable of sustained protein  
10 synthesis on these cells.

### EXAMPLE 5

#### Neurovirulence Assay

15 Wild-type HSV-1 virus, SPBg5e 34.5 deletion mutant virus, and suppressor isolate SUP1 were each diluted in DMEM plus 1% calf serum. Particulate matter was removed from viral stocks by a brief spin in a microfuge prior to dilution. Twenty-one day old female Balb/C mice (Charles River Laboratories, Massachusetts) were inoculated intracranially with 30  $\mu$ l of diluted virus. The mice, in groups of five, were injected intracranially with either 300 or 3,000 pfu of wild-type HSV-1 (wt); 300,000 pfu of SPBg5e; or 60,000 or  
20 600,000 pfu of suppressor isolate (SUP1). After injection, the mice were followed for 21 days and scored for survival. The results are illustrated in Figure 8. All the mice injected with 300 or 3,000 pfu of wild-type virus died. In contrast, the mice injected with the  
25 suppressor isolate SUP1 survived, as well as the mice injected with the non-neurovirulent 34.5 deletion mutant. The neurovirulence properties of the suppressor isolate SUP1 were identical to the neurovirulence properties of the 34.5 deletion mutant.

- 27 -

## EXAMPLE 6

Marker Rescue Analysis

Multiple, independent isolates were obtained with breakpoints clustering in the same region, suggesting that this rearrangement was a necessary component of the suppressor phenotype. To prove that it was both necessary and sufficient to confer this phenotype on  $\gamma$ 34.5 mutant viruses, marker rescue experiments were performed.

The rearranged regions in all but one SUP6 clone were contained completely between the *Bst*Ell and *Nru*I sites. These rearranged *Bst*Ell-*Nru*I fragments were then used to replace the wild-type *Bst*Ell-*Nru*I fragment in the targeting vector pSXZY. The insert in pSXZY contains HSV-1 Patton strain sequences from the *Sa*I site at nucleotide 143481 to the *Bst*Ell site at 147040. The nucleotide coordinates refer to the published sequence of HSV-1 strain 17, GENBANK accession number X14112D00317. This insert was cloned between the unique *Sa*I and *Hind*III sites in the vector pGEM9zf- (Promega, Wisconsin). As the *Bst*Ell site at 147040 and the unique *Hind*III site in pGEM9zf- were end-filled by prior treatment with the Klenow fragment of DNA polymerase I, both sites were destroyed. pSXZY thus has unique *Bst*Ell sites and *Nru*I sites to facilitate the exchange of *Bst*Ell - *Nru*I fragments from the *Bam*HI Z region. The flanking sequences, which extend in either direction from the internal *Bst*Ell - *Nru*I fragment, function to direct homologous recombination within this region of the viral chromosome. FIG. 9 illustrates the experimental approach employed to rescue the suppressor phenotype.

HSV-1 DNA from  $\gamma$ 34.5 mutant viruses was transfected either alone or cotransfected with a specific rescue plasmid into permissive Vero cells. Each rescue plasmid contained the *Bst*Ell-*Nru*I fragment from a specific suppressor isolate and could be recognized by its altered electrophoretic mobility relative to the wild type *Bam*HI Z fragment specified in the parental HSV-1  $\gamma$ 34.5 mutant, as shown in

- 28 -

FIG. 10 by comparing the  $\Delta 34.5$  lane marked C with the C lanes of SUP1, SUP3, SUP5, and SUP6. Each transfection resulted in the appearance of approximately 90 - 200 plaques on permissive Vero cells. Cell-free lysates prepared from these transfections by freeze-thawing were then used to infect non-permissive U373 glioblastoma cells. At the first appearance of cytopathic effect, a freeze-thaw lysate was again prepared and used to infect a second set of U373 cells. At this juncture, the U373 cells which were infected with lysates derived from transfections of only 34.5 viral DNA appeared as uninfected monolayers. However, U373 cells infected with lysates derived from cotransfections of 34.5 viral DNA and each specific rescue plasmid all exhibited substantial cytopathic effect (data not shown). DNA was prepared from these rescued cultures to analyze the genotype of the resulting new viruses.

FIG. 10 presents a Southern analysis of *Bam*HI Z region of the rescued isolates. In four separate instances, the rescued viruses had acquired the *Bam*HI Z fragment specified by the suppressor plasmid present in the transfection as shown in FIG. 10 by a comparison of the C and V lanes of SUP1, SUP3, SUP5, and SUP6. Only the *Bst*EII-*Nru*I fragments in each of these plasmids differed from wild-type. In the case of SUP1 this minimal fragment was 109 base pairs. The additional bands hybridizing in the  $\Delta 34.5$ , SUP1, SUP5, and SUP6 lanes are due to variation in the repetitive component of the *Bam*HI Z fragment. Similar alterations can be observed in the wild-type and SPBg5e lanes in figure 4. The slower mobility of the cloned SUP6 *Bam*HI fragment in FIG. 10 is also due to variation in a reiterated component.

The Southern analysis shown in FIG. 10 demonstrated that the viruses which have acquired the suppressor *phenotype*, assessed by cytopathic effect on U373 cells, have acquired the *genotype* specified by the specific plasmid which was co-transfected



- 29 -

with  $\gamma$ 34.5 mutant HSV-1 DNA. Viruses harboring rearranged *Bam*HI Z fragments had thus overtaken the entire population of  $\gamma$ 34.5 mutant viruses in a single passage on non-permissive cells. This high frequency generation of the suppressor phenotype in a single pass contrasted to the multiple passes needed to select these isolates.

5

- 30 -

### **CLAIMS**

1. A herpetic virus selected from the group consisting of neurotrophic herpes viruses, T-lymphotrophic viruses, and B-lymphotrophic viruses having a genome from which the gene or genes controlling virulence have been deleted and having at least one additional mutation to the genome, wherein the mutated herpetic virus is avirulent and selectively replicates in and destroys neoplastic cells.
2. The herpetic virus of claim 1, wherein the herpetic virus is herpes simplex virus-1 (HSV-1) and the deleted genes controlling virulence are the  $\gamma$ 34.5 genes.
3. The herpetic virus of claim 1, wherein the herpetic virus is herpes simplex virus-2 (HSV-2).
4. An HSV-1 deposited under ATCC accession number VR-2510.
5. A method for isolating tumoricidal herpetic viruses comprising the steps of:
  - (a) attenuating a herpetic virus to render it avirulent;
  - (b) identifying neoplastic cells which fail to support replication of the virus of step (a);
  - (c) sequentially passaging the attenuated virus of step (a) on the neoplastic cells of step (b) to produce isolates of the attenuated virus of step (a) which can replicate in the neoplastic cells of step (b); and
  - (d) isolating the viral isolates of step (c).
6. The method of claim 5, wherein the isolated virus is selected from the group consisting of neurotrophic herpes viruses, T-lymphotrophic viruses, and B-lymphotrophic viruses.
7. The method of claim 5, wherein the neurotrophic herpes viruses are selected from the group consisting of HSV-1, HSV-2, and VZV, the T-lymphotrophic virus is HHV-6, and the B-lymphotrophic virus is Epstein-Barr Virus.

- 31 -

8. The method of claim 5, wherein the isolated virus is the virus of claim 2.

9. A method for selectively destroying neoplastic cells in a mammal comprising administering to the mammal an effective amount of the isolated tumoricidal herpetic virus of claim 5.

10. The method of claim 9, wherein the isolated tumoricidal virus is the virus of claim 8.

11. The method of claim 10, wherein the neoplastic cells are glioma cells.

12. The method of claim 9, wherein the isolated tumoricidal herpetic virus is an HSV-1 virus.

13. The method of claim 9, wherein the isolated tumoricidal herpetic virus is an HSV-2 virus.

14. The method of claim 9, wherein the isolated tumoricidal herpetic virus is the HSV-1 deposited under ATCC accession number VR-2510.

15. A pharmaceutical composition comprising the herpetic virus of claim 1 and a pharmaceutically acceptable carrier, adjuvant or diluent.

16. The pharmaceutical composition of claim 15, wherein the herpetic virus is an HSV-1 virus.

17. The pharmaceutical composition of claim 15, wherein the herpetic virus is an HSV-2 virus.

18. The pharmaceutical composition of claim 15, wherein the herpetic virus is the HSV-1 virus deposited under ATCC accession number VR-2510.

19. A vaccine comprising the herpetic virus of claim 1 and a pharmaceutically acceptable diluent, adjuvant or carrier.

20. The vaccine of claim 19, wherein the herpetic virus is an HSV-1 virus.

21. The vaccine of claim 19, wherein the herpetic virus

- 32 -

is an HSV-2 virus.

22. The vaccine of claim 20, wherein the herpetic virus is an HSV-1 virus deposited under ATCC accession number VR-2510.

23. A method for immunizing a mammal against a herpetic virus comprising the step of inoculating the mammal with an immunity-inducing dose of the vaccine of claim 21 and a pharmaceutically acceptable diluent, adjuvant or carrier.

24. The method of claim 23, wherein the herpetic virus is an HSV-1 virus.

25. The method of claim 23, wherein the herpetic virus is an HSV-2 virus.

26. The method of claim 24, wherein the herpetic virus is an HSV-1 virus deposited under ATCC accession number VR-2510.

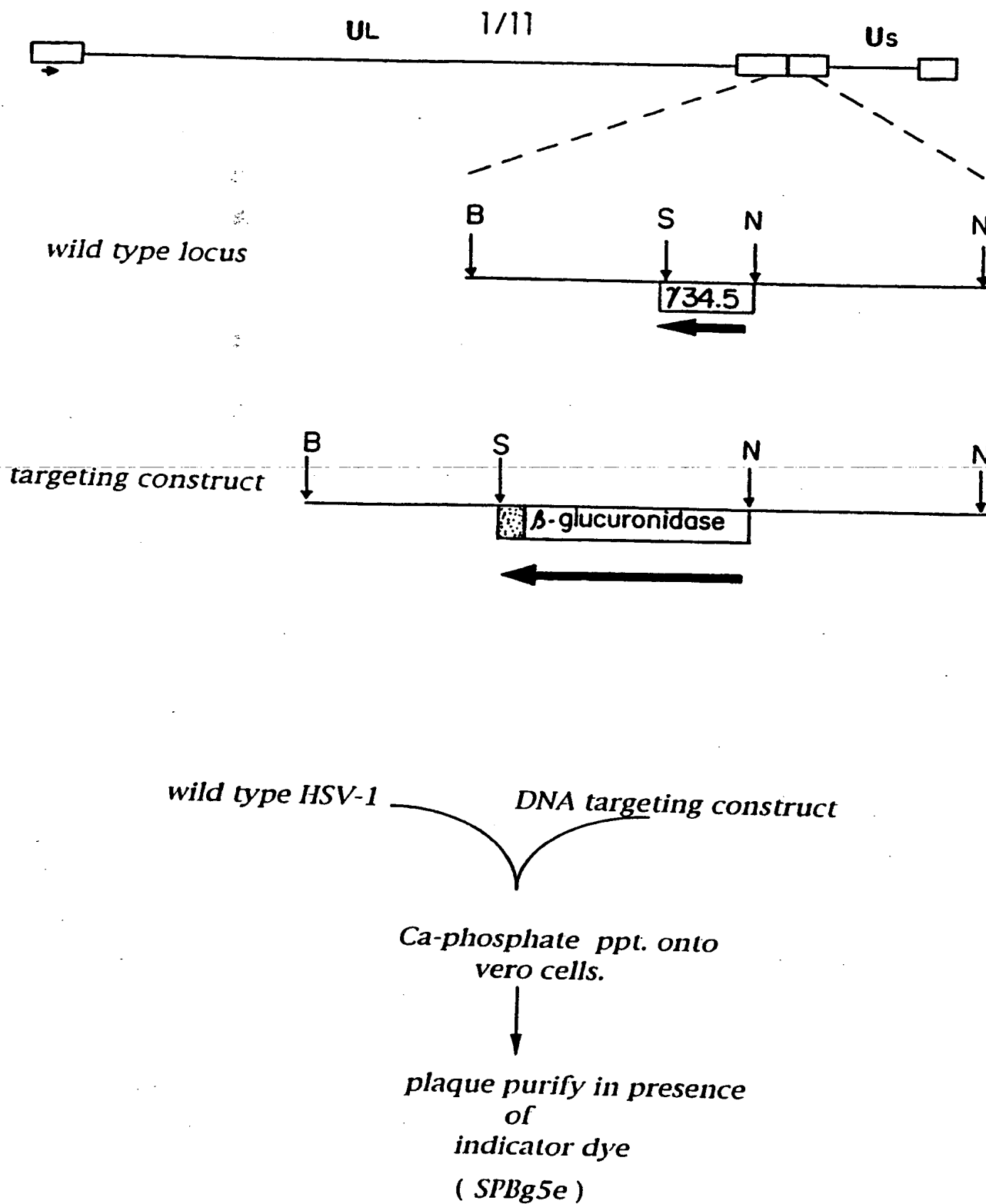


FIG.1

2/11

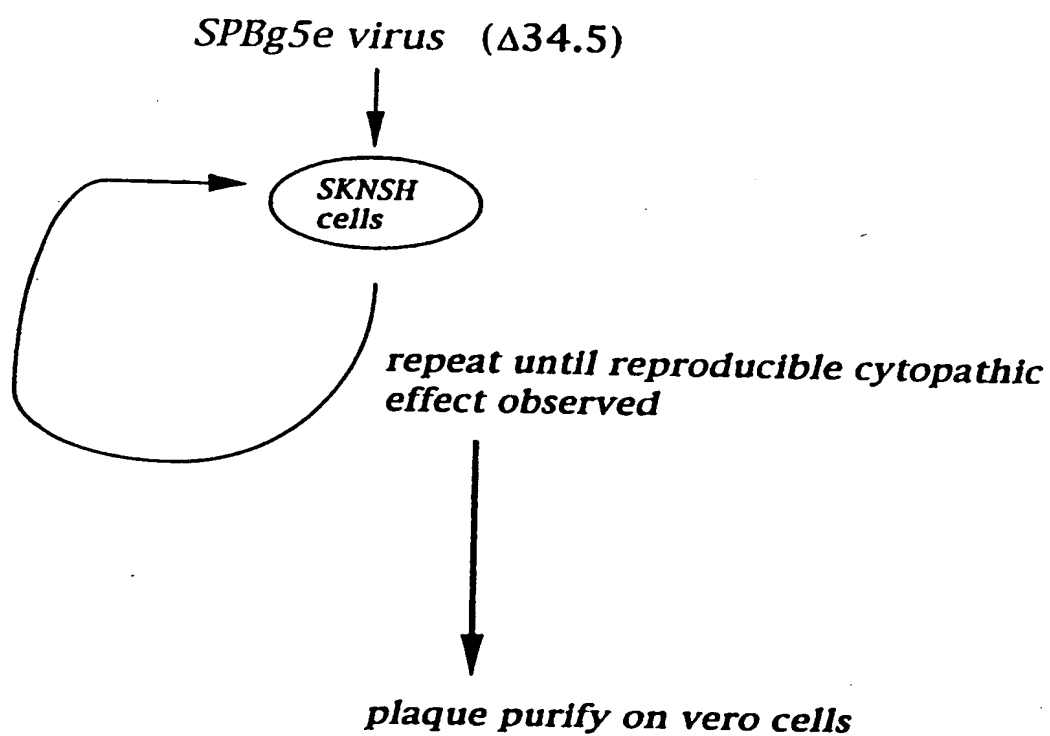
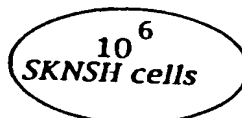


FIG. 2

3/11

*10, 100, or 1000 pfu virus**48 hrs**freeze - titer on Vero*

	<u>input pfu</u>	<u>yield SKNSH</u>
<i>Δ 34.5</i>	10	--
	100	10
	1000	125
<i>REV 4</i>	10	40
	100	1040
	1000	6400
<i>wt</i>	10	12,000
	100	$5.6 \times 10^5$

FIG. 3

4/11

W.T. HSV-1 Patton  
SPBg5e  
REV4  
1 2 3 4 5 6 7 8 10

← *Suppressor Isolates* →

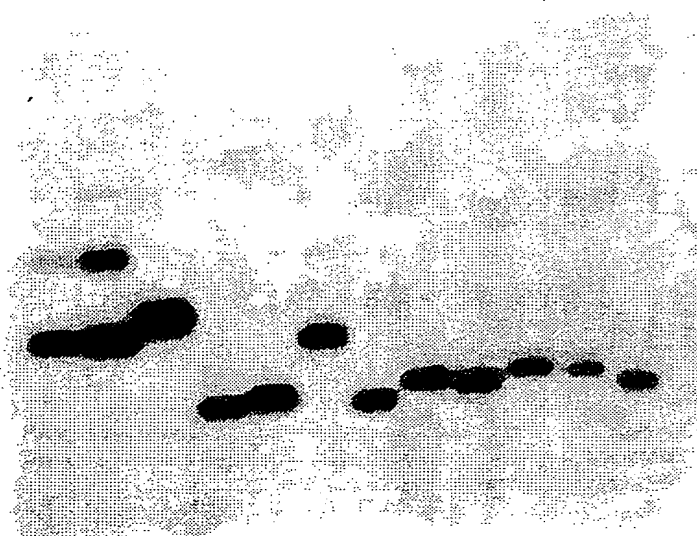


FIG.4



5/11

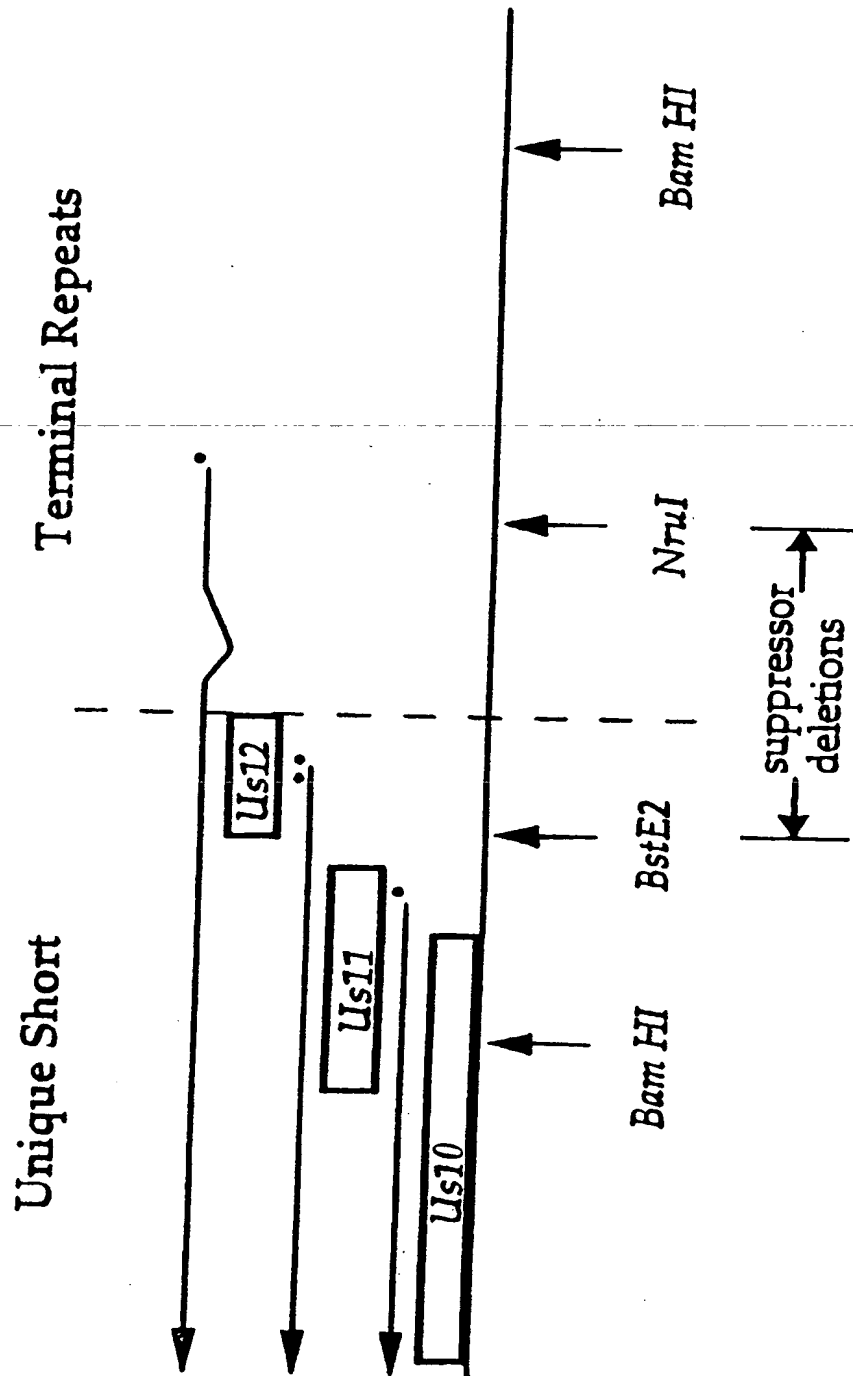


FIG. 5

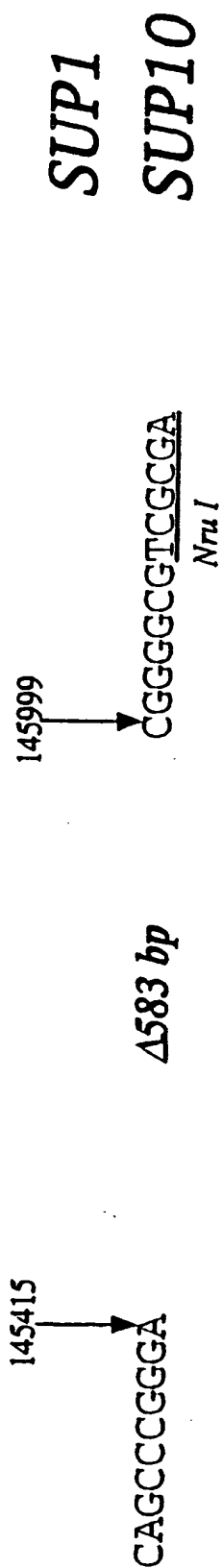


FIG. 6A

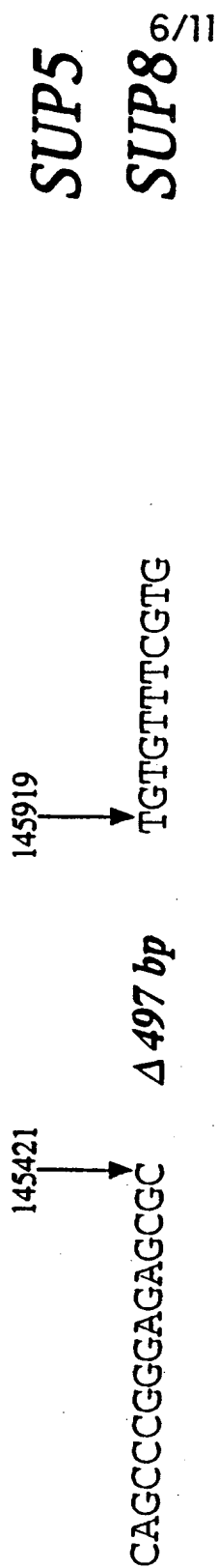


FIG. 6B

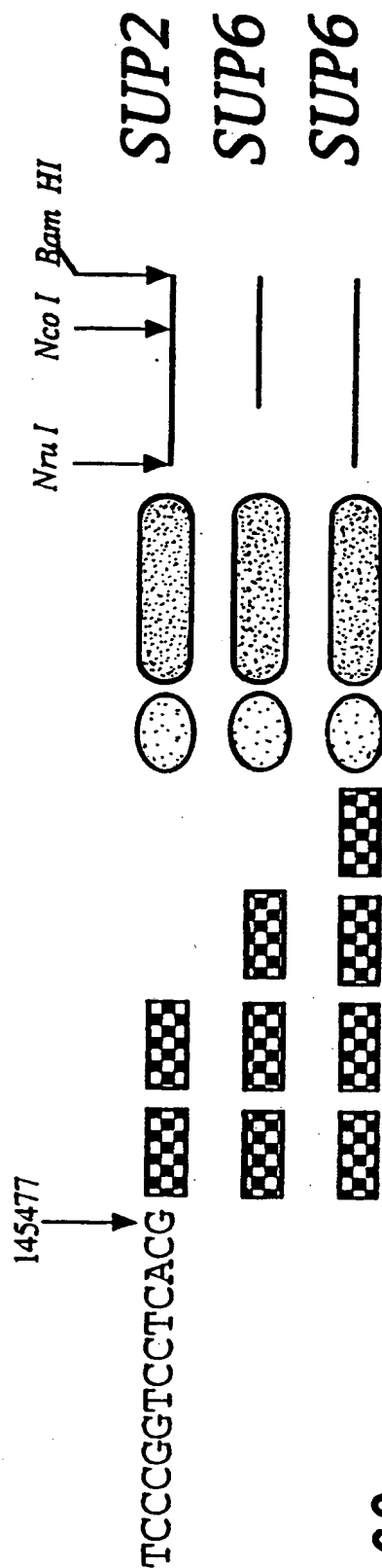
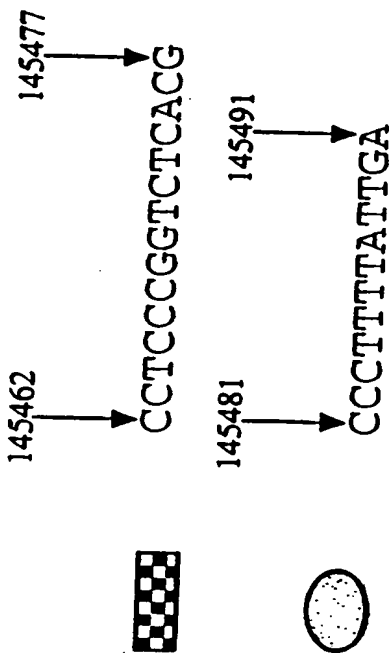
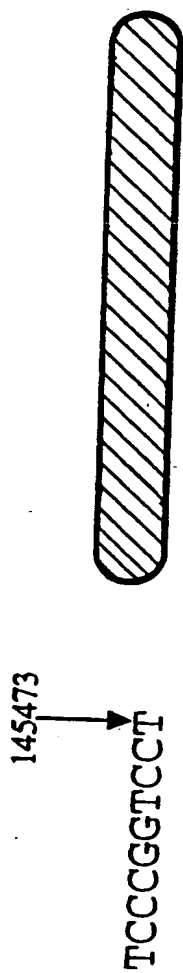
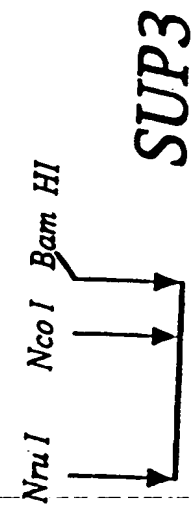


FIG. 6C

7/11



Homology to HSV -1 sequences in repeated regions

**FIG. 6D**



8/11

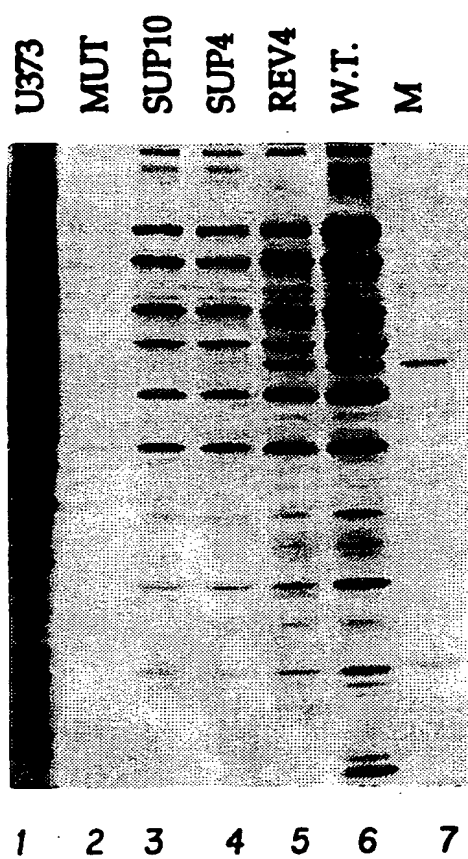


FIG.7

9/11

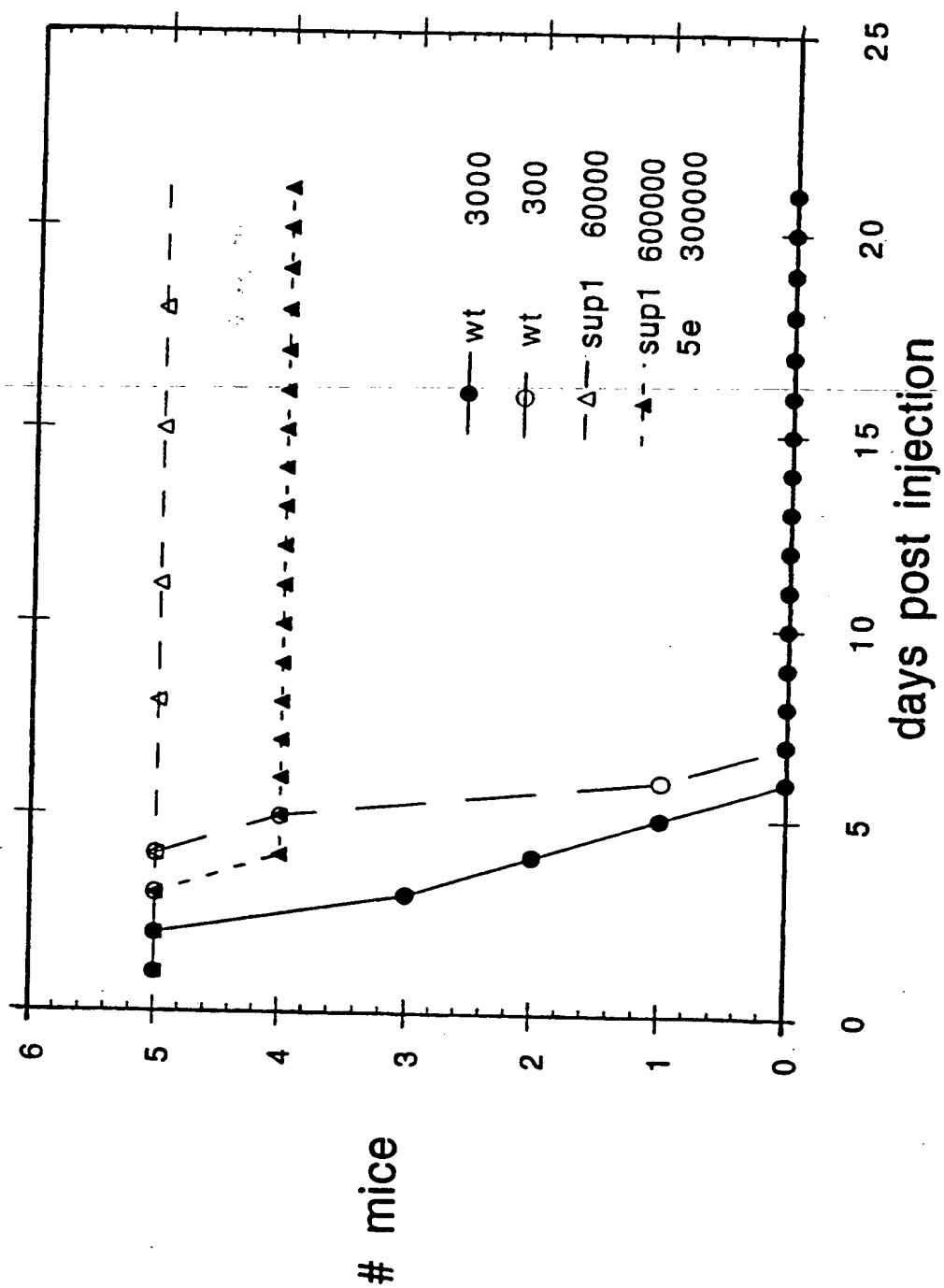


FIG. 8

10/11

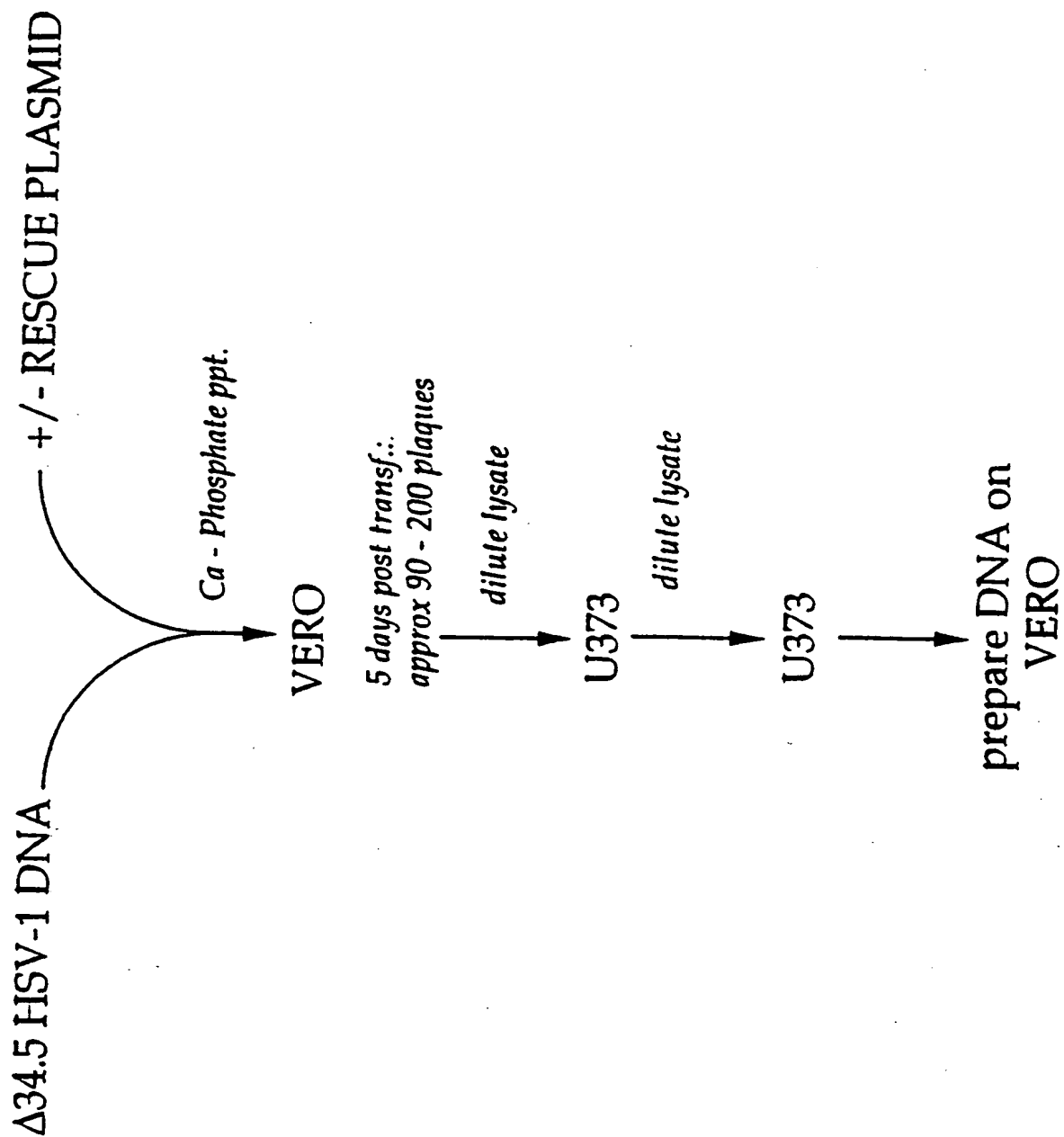


FIG. 9

11/11

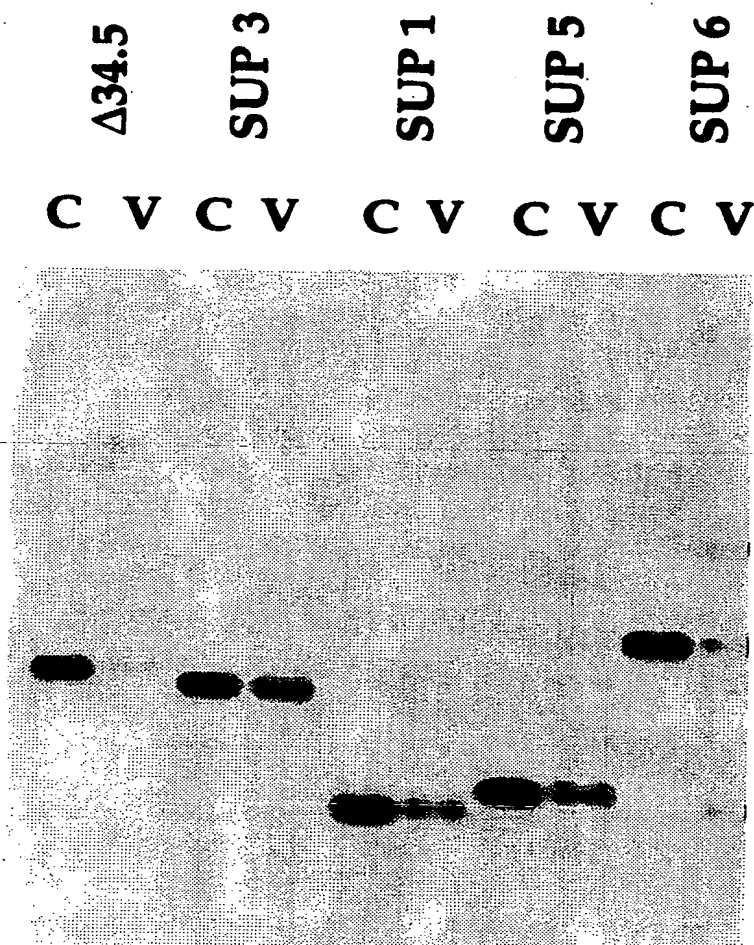


FIG.10

# INTERNATIONAL SEARCH REPORT

Intern. Application No  
PCT/US 96/12281

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K39/12 A61K35/76 C12N7/04 C12N7/08 C07K14/03

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO,A,96 00007 (GEORGETOWN UNIVERSITY) 4 January 1996 cited in the application see the whole document ---	1-26
X,P	WO,A,96 03997 (MEDICAL RESEARCH COUNCIL (GB); THE WISTAR INSTITUTE (US)) 15 February 1996 see page 6, line 16 - page 7, line 1; claims 1-18; examples 4,5 --- -/--	1-26

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*Z\* document member of the same patent family

Date of the actual completion of the international search

14 November 1996

Date of mailing of the international search report

06.12.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+31-70) 340-3016

Authorized officer

Olsen, L



# INTERNATIONAL SEARCH REPORT

Intern al Application No  
PCT/US 96/12281

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NEUROSURGERY, vol. 32, no. 4, 1 April 1993, pages 597-603, XP000561128 MARKERT, J. M. ET AL: "REDUCTION AND ELIMINATION OF ENCEPHALITIS IN AN EXPERIMENTAL GLIOMA THERAPY MODEL WITH ATTENUATED HERPES SIMPLEX MUTANTS THAT RETAIN SUSCEPTIBILITY TO ACYCLOVIR" cited in the application see the whole document ---	1-26
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE, USA, vol. 89, 1992, pages 3266-3270, XP000608916 CHOU, J. AND B. ROIZMAN: "The gamma-1 34.5 gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neural cells" cited in the application see the whole document ---	1-26
A	SCIENCE, vol. 250, 1990, pages 1262-1266, XP002018560 CHOU, J. ET AL.: "Mapping of herpes simplex virus-1 neurovirulence to gamma-1 34.5, a gene nonessential for growth in culture" see the whole document ---	1-26
A	US,A,5 328 688 (ROIZMAN, BERNARD) 12 July 1994 cited in the application see the whole document -----	1-26

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/12281

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9600007	04-01-96	AU-A- 2906095	19-01-96
WO-A-9603997	15-02-96	AU-A- 3119995	04-03-96
US-A-5328688	12-07-94	AU-B- 658838	04-05-95
		AU-A- 8741891	30-03-92
		CA-A- 2072627	11-03-92
		EP-A- 0500917	02-09-92
		JP-T- 5503017	27-05-93
		WO-A- 9204050	19-03-92